

This Page Is Inserted by IFW Operations  
and is not a part of the Official Record

## **BEST AVAILABLE IMAGES**

Defective images within this document are accurate representations of the original documents submitted by the applicant.

Defects in the images may include (but are not limited to):

- BLACK BORDERS
- TEXT CUT OFF AT TOP, BOTTOM OR SIDES
- FADED TEXT
- ILLEGIBLE TEXT
- SKEWED/SLANTED IMAGES
- COLORED PHOTOS
- BLACK OR VERY BLACK AND WHITE DARK PHOTOS
- GRAY SCALE DOCUMENTS

**IMAGES ARE BEST AVAILABLE COPY.**

**As rescanning documents *will not* correct images,  
please do not report the images to the  
Image Problem Mailbox.**



09/998.130 12.18.93



Europäisches Patentamt  
Eur pean Patent Office  
Office européen des brevets



(11) Publication number: **0 437 373 A1**

(12)

# EUROPEAN PATENT APPLICATION

(21) Application number: 91300201.0

(51) Int. Cl.<sup>5</sup>: **C12Q 1/26, C12Q 1/32,  
C12N 9/04, C12N 1/20,  
// (C12N9/04, C12R1:05)**

(22) Date of filing: 11.01.91

(30) Priority: 11.01.90 JP 4063/90  
09.11.90 JP 305439/90

(43) Date of publication of application:  
17.07.91 Bulletin 91/29

(84) Designated Contracting States:  
DE FR GB IT

(71) Applicant: TOYO JOZO KABUSHIKI KAISHA  
632-1 Mifuku Ohito-cho  
Tagata-gun Shizuoka-ken (JP)

(72) Inventor: Takahashi, Mamoru  
684-1, Kakita, Shlmizu-cho  
Sunto-gun, Shizuoka (JP)  
Inventor: Ueda, Shigeru  
1421-237, Nanjo, Nirayama-cho  
Tagata-gun, Shizuoka (JP)

(74) Representative: Woods, Geoffrey Corlett et al  
J.A. KEMP & CO. 14 South Square Gray's Inn  
London WC1R 5LX (GB)

(54) High sensitive assay method of L-carnitine and composition for an assay.

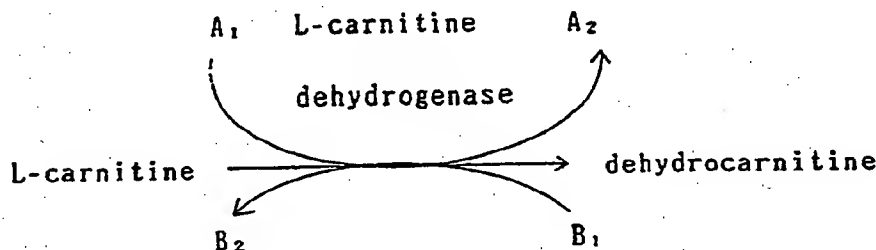
(57) A method of assaying L-carnitine which comprises reacting a specimen comprising the L-carnitine to be assayed with reagents comprising:

① L-carnitine dehydrogenase which has coenzymes of the thionicotinamide adenine dinucleotide group (hereinafter designated as the thio-NAD-group) and nicotinamide adenine dinucleotide group (hereinafter designated as the NAD group) and which catalyses a reversible reaction forming essentially dehydrocarnitine from L-carnitine;

② A<sub>1</sub>; and

③ B<sub>1</sub>;

to perform the cycling reaction:



wherein:

A<sub>1</sub> is the thio-NAD group or the NAD group;

A<sub>2</sub> is a reduced form of A<sub>1</sub>;

when A<sub>1</sub> is the thio-NAD group, B<sub>1</sub> is a reduced NAD group and when A<sub>1</sub> is the NAD group, B<sub>1</sub> is a reduced thio-NAD group, and

B<sub>2</sub> is an oxidized form of B<sub>1</sub>;

and measuring the amount of A<sub>2</sub> generated or B<sub>1</sub> consumed.

EP 0 437 373 A1

## HIGH SENSITIVE ASSAY METHOD OF L-CARNITINE AND COMPOSITION FOR AN ASSAY

Field of the Invention

This invention relates to a high sensitive assay method of L-carnitine which is especially L-carnitine in a specimen, and a composition for assaying L-carnitine.

5 The prior arts

L-carnitine is an essential substance for mediating a long-chain fatty acid transport through mitochondrian membrane prior to intracellular  $\beta$ -oxidation, and hence a deficiency of L-carnitine causes disorders in fatty acid and its related metabolisms. Particularly, it is believed that disorders of the skeletal muscle and cardiac muscle, both of which are high energy consumption tissues depending on carnitine and lacking in carnitine generation, occur from such deficiency. Heretofore a disease arising from inborn irregularities of carnitine metabolism has been studied, however in recent time, secondary disorders of carnitine metabolism have become a problem in patients suffering from nephrosis and undergoing dialysis. Carnitine is administered to carnitine deficient patients who have a disease of the body muscle or cardiac muscle, or patients undergoing dialysis. Studies on the behavior of carnitine in disease and therapy have been required, however a desirable assay method for carnitine in the clinical field has not been developed.

known assay methods for carnitine are as follows:

1. L-carnitine and acetyl CoA are treated with carnitine acetyltransferase (CAT), and the thus-liberated CoASH and 5, 5'-dithio-bis-2-nitrobenzoate (DTNB) are further reacted to generate thiophenolate ion which is colorimetrically measured (DTNB method). This method is described in J. Biol. Chem., Vol. 238, p. 2509 (1963), J. Lipid Res., Vol. 5, pp. 184-187 (1964) and Clinical Pathology, Vol. 36, N. 11, pp. 1296-1302 (1988).
2. L-carnitine and  $^{14}\text{C}$  - or  $^3\text{H}$  - labelled acetyl CoA are treated with CAT to generate labelled acetyl-L-carnitine and CoASH, and radio-activity is measured (radiolotope method). This method is described in Clin. Chem. Acta, Vol. 37, pp. 235-243 (1972), J. Lipid Res., Vol. 17, pp. 277-281 (1976), and J. Japan. Nut. Food. Soc., Vol. 41, N. 5, pp. 389-395 (1988).
3. L-carnitine and  $\text{NAD}^+$  are treated with L-carnitine dehydrogenase to generate 3-dehydrocarnitine and NADH, and increased UV absorption of NADH is measured (carnitine dehydrogenase method). This method is described in Eur. J. Biochem., Vol. 6, pp. 196-201 (1968), ibid. Vol. 10, pp. 56-60 (1969), and Fresenius Z. Anal. Chem., Vol. 320, N. 3, pp. 285-289 (1985).
4. L-carnitine and acetyl CoA are treated with CAT to generate CoA which is reacted with n- {p-(2-benzimidazolyl)-phenyl} -malimide (BIPM), and fluorescent intensity of the resulting CoA-BIPM is measured (fluorescence method). This method is described in Ann. Rep. MHW Institute for Nerve Disease, pp. 315-318 (1986).

35 Problems to be solved by the invention

In the prior arts, the DTNB method and the fluorescence method are essentially required a treatment of deproteinization in an assay of serum L-carnitine, which causes a complex operation, and radioisotope method has an advantages in its sensitivity and specificity, however special facilities are required for measuring radioactivity. The carnitine dehydrogenase method has disadvantage due to small molecular absorption coefficient of NADH, i.e.  $\epsilon = 6.22 \text{ (cm}^2/\mu\text{mol)}$  at 340 nm, and hence it is difficult to assay serum carnitine in a patient of carnitine deficient disease [Neurology, 25: 16-24(1975)]; moreover generated NADH is consumed by another dehydrogenase in serum such as lactate dehydrogenase, which causes an error in measurement.

We had invented previously an assay method of L-carnitine, in which generated formazane in an enzymatic reaction with L-carnitine dehydrogenase was quantitatively measured (Japan.Pat. Appln. No 1-196550). The said methods has, however, disadvantages with having insufficient sensitivity for a small amount of serum which being collected from prematured infant such as an amount of 20  $\mu\text{l}$  as compared with relatively large amount of serum from adult.

Under these circumstances, it has been desired to develop advantageous method for assaying L-carnitine without required complex treatment such as deproteinization and specific facilities and with having possibility for measuring trace amount of serum L-carnitine of prematured infant.

50 Description of the invention for solving the problem

We have studied a reaction system using L-carnitine dehydrogenase (EC 1.1.1.108), and found that, in a

reversible reaction in which dehydrocarnitine was generated from substrate L-carnitine, when a reaction system, wherein dehydrocarnitine was generated from L-carnitine with a coenzyme of NAD group, together with another trace amount NADH group of coenzyme, was subjected to reversible cycling reaction between L-carnitine and dehydrocarnitine, a linear increase of generated amount of NADH group was observed depending on a time course, furthermore an increasing rate thereof was in proportion to an amount of L-carnitine or dehydrocarnitine.

We have further found that in the said enzymatic cycling reaction, thionicotinamide adenine dinucleotide group (hereinafter designates as thio-NAD group) or reduced from thio-NAD group (hereinafter designates as thio-NADH group) is used in NAD group or NADH group, and an amount of changes in any of coenzymes is measured depending upon a difference in a maximum absorption of NADH group at approx. 340 nm and that of thio-NADH group at approx. 400 nm, an amount of L-carnitine or dehydrocarnitine can precisely be measured.

### Objects of the invention

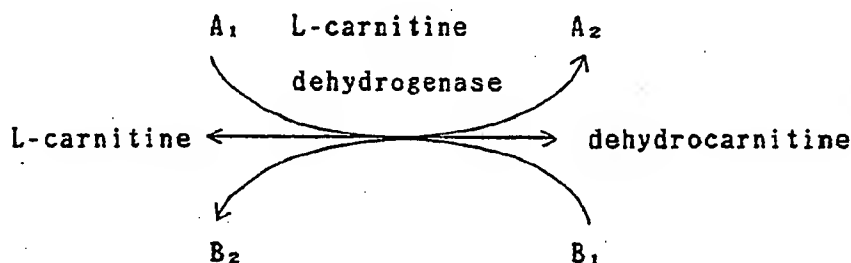
An object of the present invention is to provide a high sensitive assay method of L-carnitine which comprises reacting a specimen with reagents containing

- ① L-carnitine dehydrogenase which has coenzymes of thionicotinamide adenine dinucleotides group (hereinafter designates as thio-NAD group) and nicotinamide adenine dinucleotides group (hereinafter designates as NAD group) and which catalyses a reversible reaction with forming essentially dehydrocarnitine from a substrate L-carnitine,

②  $A_1$  and

③  $B_1$

to construct a cycling reaction of the formula



wherein  $A_1$  is thio-NAD group or NAD group,  $A_2$  is a reduced form of  $A_1$ , when  $A_1$  is thio-NAD group,  $B_1$  is reduced NAD group and when  $A_1$  is NAD group,  $B_1$  is reduced thio-NAD, and  $B_2$  is oxidized form of  $B_1$  and measuring an amount of  $A_2$  or  $B_1$  which depends on the said reaction.

Another object of the present invention is to provide a composition for assaying L-carnitine which comprises containing essentially the following components ① - ③

- ① L-carnitine dehydrogenase which has coenzymes of thionicotinamide adenine dinucleotides group (hereinafter designates as thio-NAD group) and nicotinamide adenine dinucleotides group (hereinafter designates as NAD group) and which catalyses a reversible reaction with forming essentially dehydrocarnitine from a substrate L-carnitine,

②  $A_1$  and

③  $B_1$

wherein  $A_1$  is thio-NAD group or NAD group, when  $A_1$  is thio-NAD group,  $B_1$  is reduced form of NAD group, and when  $A_1$  is NAD group,  $B_1$  is reduced form of thio-NAD group.

### Brief Description of the Drawings

Figure 1 : Rate assay on an amount of L-carnitine at 400 nm in Example 1.

Figure 2 : Rate assay on an amount of serum at 400 nm in Example 2.

In the present invention L-carnitine dehydrogenase can be used in any type of the said enzyme having the properties hereinabove. Examples of L-carnitine dehydrogenase used in the present invention are L-carnitine dehydrogenase produced by the following microorganisms.

*Pseudomonas aeruginosa* A 7244 (NCTC) [Eur. J. Biochem., Vol. 6, pp. 196-201 (1968), *ibid.*, Vol. 10, pp.

56-60 (1969)] ;

Pseudomonas putida IFP 206 (Arch. Microbiol., Vol. 116, pp. 213-220 (1978), Biochem. Biophys. Acta, Vol. 957, pp. 335-339 (1988) ;

Pseudomonas putida ATCC 17633 [Fresenius' Z. Anal. Chem., Vol. 320, pp. 285-289 (1985)] ; and

Xanthomonas translucens IF0 13558 [Agr. Biol. Chem., Vol. 52, pp. 851-852 (1988)] .

Alcaligenes sp. No. 981 FERM BP-2570 [Product of Toyo Jozo Co., Japan. Pat. Appln. No. 1-267919]

Among these, L-carnitine dehydrogenase originated from Alcaligenes sp. No. 981 is preferable in its stability in a buffer solution.

L-carnitine dehydrogenase originated from Alcaligenes sp. No. 981 is a novel L-carnitine dehydrogenase, which is produced, for example, by a microorganisms of the genus Alcaligenes sp. No. 981 FERM BP-2570, isolated from a soil sample from a potato field in Gojo-shi, Nara prefecture, Japan.

#### Detailed Description of the Invention

The taxonomical properties of this strain are illustrated as follows :

##### A. Morphological properties :

Observations on a nutrient agar medium, cultures for 18-24 hours at 28-30°C, are as follows.

Round edge with straight or slightly curved bacillus and single or double linked somewhat short chain. No formation of spores. Sizes are 0.4-0.6 X 1.2-2.5 µm. Peritrichal movement. No polymorphism.

##### B. Growth on various media :

Observations on various media, cultured for 18-24 hours at 28-30 °C, are as follows.

##### 1. Nutrient agar slant medium :

Good growth with filiform.

Wetish with luminescence. Ocherous but no formation of soluble pigment.

##### 2. Nutrient agar plate medium :

Round, convex and whole round colonies. Smooth wetish surface. Ocherous or pale ocherous.

No formation of soluble pigment.

##### 3. Liquid medium (Aqueous peptone) :

Good growth with uniform turbidity. Formation of pellicle at long term (over 40 hours) culture.

##### 4. BCP milk medium :

Alkaline after 4-5 days.

C. Physiological properties (+ = positive, ( + ) = weekly positive, - = negative )

5

Gram-stain -

KOH reaction +

10

Capsule formation -

Acid fastness stain -

OF-test (Hugh Leifson) No change

15

OF-test (nitrogen source:  $\text{NH}_4\text{H}_2\text{PO}_4$ ) 0 (oxidative)

Aerobic growth +

20

25

30

35

40

45

50

55

EP 0 437 373 A1

	Anaerobic growth	—
	Growth temperature 41°C	—
5	37°C	+
	15°C	+
10	Halotolerant NaC 2 conc. %	
	0 %	+
15	5 %	+
	7 %	—
	Growth pH pH 4.6	—
20	pH 5.4	+
	pH 8.9	+
25	pH 9.8	—
	Gelatin hydrolysis	—
	Starch hydrolysis	—
30	Casein hydrolysis	—
	Esculin hydrolysis	—
35	Cellulose hydrolysis	—
	Tyrosine hydrolysis	—
	Catalase production	+
40	Oxidase production	+
	LV-reaction	—
45	Urease production (SSR)	—
	Urease production (Chris)	—
	Indol production	—
50		
55		



H<sub>2</sub>S production (detection:

lead acetate paper)

—

5

Acetoin production (K<sub>2</sub>HPO<sub>4</sub>)

—

Acetoin production (NaCl)

—

10

MR test

—

## Nitrate reduction

15

Gas detection

+

NO<sub>2</sub> - detection

—

20

NO<sub>3</sub> - detection

—

## Utilization on Simmons medium

25

Citrate

+

Malate

+

30

Maleate

—

Malonate

( + )

Propionate

—

35

Glucosate

—

Succinate

+

40

## Utilization on Christenssen medium

Citrate

+

45

Malate

+

Maleate

+

Malonate

+

50

Propionate

—

55

	Gluconate	+
	Succinate	+
5	Gas production from glucose	-
	Acid formation from sugar	
	Adonitol	-
10	L(+) arabinose	(+)
	Cellobiose	-
15	Dulcitol	-
	Meso-erythritol	-
	Fructose	-
20	Galactose	+
	Glucose	+
25	Glycerin	(+)
	Inositol	-
	Inulin	-
30	Lactose	-
	Maltose	-
35	Mannitol	-
	Mannose	+
	Melezitose	-
40	Melibiose	-
	Raffinose	-
45	L(+) rhamnose	-
	D-ribose	-
	Salicin	-
50	L-sorbose	-

55

	Sorbitol	—
	Starch	—
5	Saccharose	—
	Xylose	—
10	Trehalose	—
	Poly- $\beta$ -hydroxybutyrate accumulation	—

## D. Utilization of carbon sources :

15 Test medium : liquid medium (pH 7.0) containing carbon source 5 g, NaCl 5 g,  $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$  0.2 g,  $\text{NH}_4\text{H}_2\text{PO}_4$  1.0 g and distilled water 1 l. Results are as follows :

20	Glucose	+
	L(+) arabinose	—
	Fructose	+
25	Mannitol	—
	Mannose	+
	Gluconate	+
30	Acetate	+
	Adipate	—
35	Pimrate	+
	Suberate	+
40	Tartrate	+

40 According to the above taxonomical properties, the microorganism displays the specific characteristics of Gram negative bacillus, namely, it is peritrichal in movement, is catalase positive and oxidase positive, does not produce acid from glucose in Hugh-Leifson medium containing pepton, and promotes oxidative decomposition of glucose and acid formation. It displays no spore formation nor polymorphism, and is aerobic.

45 Among Gram-negative bacillus, there are three microorganisms genera which are peritrichal in movement, namely Alcaligenes, Chromobacterium and Flavobacterium. Chromobacterium produces violet colored pigment, and Flavobacterium produces yellow colored pigment ; however, the present strain does not produce pigment. Hence the present strain belongs to the genus Alcaligenes.

50 Taxonomic properties of Alcaligenes in comparison with those of the present strain according to Bergey's Manual of Systematic Bacteriology, Vol. 1 (1984), are illustrated with comparing Alcaligenes faecalis (hereinafter designated as F), Alcaligenes denitrificans subsp. denitrificans (hereinafter designated as D) and Alcaligenes denitrificans subsp. xylosoxidans (hereinafter designated as X), as follows :

+ = positive probability over 90 % .

— = negative probability over 90 % .

55 d = not identified as + or —.

	<u>F</u>	<u>D</u>	<u>X</u>	<u>The present Strain</u>
5				
	Oxidase production	+	+	+
	Nitrate reduction	-	+	+
	Nitrite reduction	+	+	+
10	Gelatin hydrolysis	-	-	-
15	Acid formation in OF-medium			
	Xylose	-	-	+
	Glucose	-	-	+
20	Acid formation in peptone-free medium			
	Xylose		+	-
	Glucose		+	+
30	Utilization of carbon sources			
	Glucose	-	-	+
35	L.(+) arabinose	-	-	-
	Fructose	-	-	d
	Mannitol	-	-	-
40	Mannose	-	-	d
	Gluconate	-	+	+
45	Acetate	+	+	+

According to the above comparison, the present strain No.981 has many identical properties with Alcaligenes denitrificans subsp. xylosoxidans but has specific differences as to acid formation in OF-medium and acid formation from xylose. Accordingly, the present strain has been designated Alcaligenes sp. No. 981 and has been deposited at Fermentation Research Institute and assigned No. FERM BP-2570.

In the enzymatic reaction hereinbefore illustrated, A<sub>1</sub> or B<sub>2</sub> is thio-NAD group or NAD group of coenzymes. Examples of NAD group are nicotinamide adenine dinucleotide (NAD), acetylpyridine ad nine dinucleotide (acetyl NAD), acetylpyridine hypoxanthine dinucleotide and nicotinamid hyp xanthine dinucleotide (deamino NAD). Examples of thio-NAD group are thionicotinamide adenine dinucleotid (thio-NAD) and thionicotinamide hypoxanthine dinucleotide

In the present invnetion, when A<sub>1</sub> is thio-NAD group, B<sub>1</sub> is NAD group, and when A<sub>1</sub> NAD group, B<sub>1</sub> is thio-NADH. Hence at least the one should be thio-type coenzyme.

Amounts of A<sub>1</sub> and B<sub>1</sub> is being excess as compared with that of L-carnitine and is essentially being excess

as compared with  $K_m$ -value of carnitin dehydrogenase for  $A_1$  and  $B_1$ , and specifically 20-10,000 molar excess of an amount of carnitine is preferable.

In a composition for assay of L-carnitine of the present invention, concentration of  $A_1$  and  $B_1$  is 0.02-100 mM, preferably 0.05-30 mM, and a concentration of L-carnitine dehydrogenase is 5-1000 U/ml, preferably 10-150 U/ml, or more.

L-carnitine dehydrogenase used in the composition for assay of L-carnitine of the present invention can be an enzyme having reactivity on a substrate L-carnitine together with preferable coenzyme NAD or thio-NAD. It can be confirmed by using the said coenzyme and substrate. L-carnitine dehydrogenase produced by *Alcaligenes* sp. No. 981 (product of Toyo Jozo) has relative activity of approx. 15 % when coenzyme thio-NAD is used as compared with NAD.  $K_m$ -value on L-carnitine, NAD and thio-NAD at the same condition is 9.3 mM, 0.14 mM and 0.40 mM, respectively.

In a composition of reaction medium, two kinds of coenzymes are selected by considering relative activity of L-carnitine dehydrogenase on each coenzyme, thereafter pH condition thereof on each optimum pH of the forward reaction and reverse reaction is adjusted to setup the pH-condition wherein a ratio of reaction rate on the forward reaction and reverse reaction reaches to 1.

In the present invention, L-carnitine dehydrogenase from single origin or plural origins can be used.

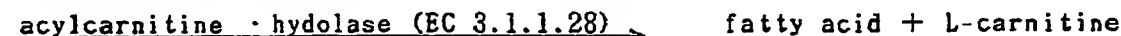
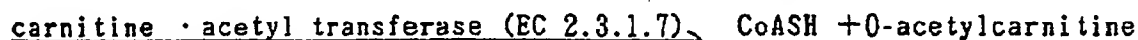
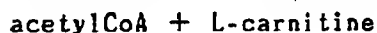
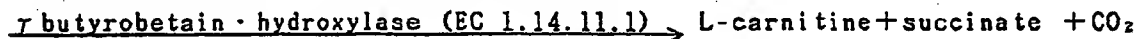
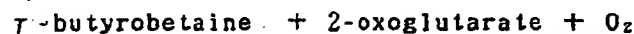
L-carnitine in a specimen can be assayed by adding a specimen 0.001-0.5 ml to the composition for assay containing the above components ① - ③, reacting at 37°C, then measuring an amount of generated  $A_2$  or consumed  $B_1$  on an interval of the two points after starting the reaction, for example one minute between 3 minutes and 4 minutes after starting, or 5 minutes between 3 minutes and 8 minutes after starting the reaction, by determining the changes of absorption at each optical absorption. Alternatively, an enzymatic reaction is stopped after constant time of starting the reaction, for example after 10 minutes, then changes of absorption value can be measured. For example, in the case that  $A_2$  is thio-NADH and  $B_1$  is NADH, a generating  $A_2$  is measured by an increase of absorption at 400 nm [molecular absorption coefficient :  $11,200 \text{ M}^{-1}\text{cm}^{-1}$  (Methods in Enzymology. Vol. 55, p. 261 (1979))] or a consuming  $B_1$  is measured by a decrease of absorption at 340 nm (molecular absorption coefficient :  $6220 \text{ M}^{-1}\text{cm}^{-1}$ ), then the thus obtained value is compared with the value of known concentration of authentic L-carnitine, thereby a concentration of L-carnitine in a specimen can be measured with real-time.

According to an assay method of the present invention, since L-carnitine per se in a specimen is introduced into an enzymatic cycling reaction, it is hard to be effected with coexisting substance in the specimen, and hence a measurement of blank value of specimen is not required. Therefore simple assay system by a rate assay can be achieved.

In the present invention, measuring a value of  $A_2$  or  $B_1$  by absorbancy can be replaced by the other known enzymatic methods.

Further in the assay method of the present invention, not only a free L-carnitine but also a liberated L-carnitine from hydrolysis of acyl carnitine such as acetyl carnitine can also be assayed. L-carnitine per se in a specimen can directly be assayed by an assay method of the present invention without hydrolysis of the specimen. Thereafter total amount of L-carnitine and acylcarnitine can be assayed after hydrolysing the specimen. An amount of acylcarnitine in the specimen can be obtained by that an amount of L-carnitine in the specimen without hydrolysis is reduced from a total amount of L-carnitine after hydrolysing acylcarnitine in the specimen.

Furthermore, a substrate in an enzymatic reaction system on a generation or consumption of L-carnitine, or an enzymatic activity thereof can also be assayed. Examples of these enzymatic systems are :



carnitine · dercarboxylase (EC 4.1.1.42)

carnitine  $\longrightarrow$  2-methylcholine + H<sub>2</sub>O

and in these systems, measurement of substrate or enzymatic activity can be achieved by the assay method of the present invention.

Effect of the Invention

As hereinabove explained, the present invention has advantages that no error on measurement can not be occurred due to use coenzyme having each different absorption in its reduced form, and that free L-carnitine, total carnitine and acylcarnitine can also be assayed with precisely and rapidly even by a small amount of specimen.

Examples

The following examples illustrate the present invention but are not to be construed as limiting.

Ref. Example 1(i) Culturing *Alcaligenes* sp. No. 981:

DL-carnitine hydrochloride (Sigma Chem. Co.)	3.0 %
KH <sub>2</sub> PO <sub>4</sub>	0.2 %
K <sub>2</sub> HPO <sub>4</sub>	0.4 %
MgSO <sub>4</sub> · 7 H <sub>2</sub> O	0.05 %
FeSO <sub>4</sub> · 7 H <sub>2</sub> O	0.002%
MnSO <sub>4</sub> · nH <sub>2</sub> O	0.001%
pH 7.0	

100 ml of a liquid medium comprising the above composition was sterilized in a 500 ml Erlenmeyer flask was sterilized at 120°C for 20 minutes. One loopful of *Alcaligenes* sp. No. 981 was inoculated into the medium and the medium was cultured at 28 °C with stirring at 120 rpm for 40 hours to obtain the cultured mass (95 ml)(enzyme activity : 1.2 U/ ml).

(ii) DL-carnitine hydrochloride (Sigma Chem. Co.)	3.0 %
yeast extract (Kyokuto Seiyaku Co.)	0.1 %
K <sub>2</sub> HPO <sub>4</sub>	0.054%
KH <sub>2</sub> PO <sub>4</sub>	0.746%
MgSO <sub>4</sub> · 7 H <sub>2</sub> O	0.05 %

CaCl <sub>2</sub> · 2 H <sub>2</sub> O	0.002%
FeSO <sub>4</sub> · 7 H <sub>2</sub> O (pH 7.0)	0.002%
MnSO <sub>4</sub> · n H <sub>2</sub> O	0.002%
disform CB 442 (Nihon Yushi Co.)	1 ml /lit.
pH 7.0	

20 l of a liquid medium comprising the above composition was sterilized in a 30 l jar fermenter by heating. 90 ml of the precultured seed culture obtained in step (i) above was inoculated therein and the mixture was cultured at 28 °C, with aeration of 20 l/min, inner pressure 0.4 kg/cm<sup>2</sup>, and agitation at 200 rpm for 27 hours to obtain the cultured mass (19 l). (enzyme activity : 3.0 U/ml).

#### Ref. Example 2

##### Purification of enzyme :

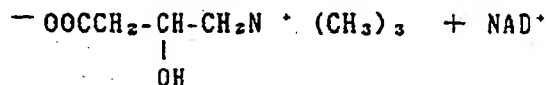
Bacterial cells collected by centrifugation from the cultured broth (19 l) obtained in Ref. Example 1, Culture (ii), were suspended in 40 mM Tris-HCl buffer (pH 8.0, 5 l) containing 0.1 % lysozyme and 15 ml EDTA-2 Na and solubilized at 37°C for 1 hour ; then the mixture was centrifuged to remove precipitate and to obtain a supernatant solution (4500 ml)(activity : 10.3 U/ml). 1100g ammonium sulfate was added to the supernatant solution, which was mixed well by stirring and then centrifuged to separate the precipitate. An additional 700 g ammonium sulfate was then added to the supernatant solution to dissolve the precipitate, and the solution was centrifuged to obtain a new precipitate. The new precipitate was dissolved in 40 mM Tris-HCl buffer (pH 8.0, 500 ml)(specific activity 84.1 U/ml), and the resultant solution was dialysed against 40 mM Tris-HCl buffer (pH 8.0, 10 lit.). The dialysed enzyme solution was charged on a column of DEAE-Sepharose CL-6B (Pharmacia Co.) (200 ml) which was buffered with 40 mM Tris-HCl buffer (pH 8.0), washed with 40 mM Tris-HCl buffer containing 0.1 M KCl, (pH 8.0, 1 lit.) and eluted with 40 mM Tris-HCl buffer containing 0.3 M KCl (pH 8.0) to obtain an enzyme solution (300 ml, specific activity 120.5 U/ml). The enzyme solution was dialysed against 40 mM Tris-HCl buffer (pH 8.0, 10 lit.). The Dialysed enzyme solution was charged on a column of hydroxylapatite (KOKEN Co., 100 ml), which was buffered with 40 mM Tris-HCl buffer, washed with 40 mM Tris-HCl buffer, washed with 40 mM Tris-HCl buffer (pH 8.0, 200 ml), then eluted with 2 mM phosphate buffer (pH 7.0, 100 ml) to obtain enzyme solution (100 ml, specific activity 331 U/ml). The thus obtained enzyme solution was dialysed against 20 mM phosphate buffer (pH 7.5, 5 lit.) to obtain 95 ml of an enzyme solution having a specific activity of 331 U/ml. The yield was 67.8 %.

The purified L-carnitine dehydrogenase was found to have an NADH oxidase activity of less than 0.0001 U/ml.

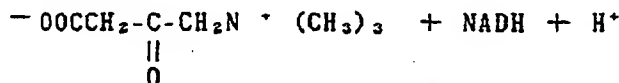
The L-carnitine dehydrogenase thus obtained has the following properties :

##### 1. Enzyme action :

The enzyme catalyses at least a reaction of L-carnitine and NAD<sup>+</sup> to generate 3-dehydrocarnitine and NADH, as shown below.



L-carnitine



3-dehydrocarnitine

## 2. Substrate specificity :

L-carnitine	100 %
Choline	0
5 Glycinebetaine	0
Glucose	0
Lysine	0

## 3. Molecular weight :

51000  $\pm$  6000

Measured by TSK-gel G3000 SW (Toso Co., 0.75 X 60 cm)

Elution : 0.1 M phosphate buffer (pH 7.0) containing 0.2 M NaCl.

Standard : following molecular markers (Oriental yeast Co.) are used.

15 M. W. 12,400	Cytochrome C
M. W. 32,000	adenylate kinase
M. W. 67,000	enolase
M. W. 142,000	lactate dehydrogenase
M. W. 290,000	glutamate dehydrogenase

## 4. Isoelectric point :

pH 5.3  $\pm$  0.6

Measured by electrofocussing using carrier ampholite at 4 °C, 700V, for 40 hours. The activity of a fraction of each enzyme is measured.

5. Km-value : 0.141 mM (NAD<sup>+</sup>), 9.3 mM (L-carnitine)

Km-value for NAD<sup>+</sup> is measured in various concentrations of NAD<sup>+</sup> in a reaction mixture of :

100 mM Tris-HCl buffer (pH 9.0)

5 U diaphorase (Toyo Jozo Co.)

0.025 % NBT (Wako Pure Chem. Co.)

1 % polyoxyethylene (20) sorbitan monooleate 80 (Wako Pure Chem., Co.) and

50 mM L-carnitine.

In the reaction mixture, 50 mM L-carnitine is replaced by 1 mM NAD<sup>+</sup>, and concentration of L-carnitine is varied to measure the Km-value of L-carnitine.

The results are as shown above.

## 6. Heat stability :

The enzyme, dissolved in 20 mM Tris-HCl buffer (pH 8.0), to produce a 1.00 U/ml solution, is incubated for one hour at various temperatures, and the residual activity is measured.

The results can be seen that the enzyme is stable up to 45°C .

## 7. Optimum temperature

The enzyme activity is measured at 35, 40, 45, 50, 55 and 60°C, respectively, in 100 mM Tris-HCl buffer (pH 9.0) according to the assay method illustrated hereinafter. The reaction was stopped in each case after 10 mins. Incubation by adding 0.1 N HCl (2 ml), whereupon the optical absorption at 550 nm. The enzyme shows maximum activity at 50°C.

## 8. pH - stability :

The residual activity of the enzyme (1 U/ml, 40 mM buffer solution) is measured in various buffer solutions, i.e. acetate buffer, pH 5.6-6.0 ; phosphate buffer, pH 6.0-8.0 ; Tris-HCl buffer, pH 8.0-9.0 and glycine-NaOH buffer, pH 9.0-10, after heating at 45 °C for 30 mins. The enzyme is stable at pH 8.0-9.0 with a residual activity of over 95 %.



## 9. Optimum pH :

In an assay method for enzyme activity as illustrated hereinafter, 100 mM Tris-HCl buffer in the reaction mixture is replaced by 100 mM phosphate buffer (pH 6.5-7.5), 100 mM Tris-HCl buffer (pH 8.0-9.0) and 100 mM glycine-NaOH buffer (pH 9.0-10.0), and incubated at 37°C for 10 mins. The reaction was stopped in each case by adding 0.1N HCl (2 ml), whereupon the absorption at 550 nm was measured.

A maximum activity is observed at approx. pH 9.0.

## 10. Long term stability in aqueous solution :

Stability of L-carnitine dehydrogenase added with 0.05 mM NAD is measured in 50 mM Tris-HCl buffer (pH 9.0, 10 U/ml) at 5 °C after two weeks storage.

L-carnitine dehydrogenase of the present invention has a residual activity of 96 % after one week and 82 % after two weeks, thus showing superior stability.

## 11. Assay method of L-carnitine dehydrogenase activity :

## (1) Reaction mixture :

50 mM Tris-HCl buffer (pH 9.0)  
1 mM NAD +  
5 U Diaphorase (Toyo Jozo Co.)  
0.05 % NBT (Wako Pure Chem. Co.)  
100 mM KCl  
0.5 % polyoxyethylene (20) sorbitan monooleate (Wako Pure Chem. Co.)  
100 mM L-carnitine (Sigma Chem. Co.)

## (2) Enzyme assay :

The above reaction mixture (1 ml) is incubated in a small test tube at 37 °C for 5 mins. Dilute enzyme solution (0.02 ml) is added and stirred to initiate the reaction. After exactly 10 mins., 0.1 N HCl (2.0 ml) was added and stirred to stop the reaction. Absorption at 550 nm ( $A_{550}$  nm) is measured to obtain absorption  $A_1$ . The assay was repeated using the above reaction mixture except that L-carnitine was not included. The mixture is also treated in the same manner as described above and its absorption  $A_0$  was measured.

## (3) Calculation of enzyme activity :

$$U/ml = \frac{(A_1 - A_0)}{21.7} \times \frac{1}{10} \times \frac{3.02}{0.02} \times Z$$

wherein

21.7 : molecular absorption coefficient  $cm^2/\mu mol$

Z : dilution ratio

Example 1

## Reagent :

100 mM Tris-HCl buffer (pH 9.5)  
5 mM Thio-NAD (Sankyo Co.)  
0.2 mM NADH (Oriental Yeast Co.)  
92 U/ml L-carnitine dehydrogenase (obtained from Ref. Example 2)

## Operation :

The above reagent (1 ml) was put into cuvettes and L-carnitine solution (0.05 ml) (0, 10, 20, 30, 40 and 50  $\mu M$ , respectively) was added therein, and started the reaction at 37 °C. After incubation was started, a difference in absorbancy at 3 mins. and 5 mins. was measured. Result is shown in Fig. 1. Linear relation between an amount of L-carnitine and changes absorption was observed.

Example 2

## Reagent :

40 mM Glycine-NaOH buffer (pH 10.0)

5 mM Thio-NAD (Sankyo Co.)  
 0.5 mM NADH (Oriental Yeast Co.)  
 0.5 % polyoxyethylene (20) sorbitan monooleate (Wako Pure Chem. Co.)  
 120 U/ml L-carnitine dehydrogenase (obtained from Ref. Example 2)  
 5 2 mM Oxamic acid

Operation :

The above reagent (1 ml) was put into cuvette. Five-fold dilution of normal serum (each 50  $\mu$ l) was added therein, and incubated at 37 °C. After reaction was started, absorbancy at 400 nm was measured at 1 min. and 6 min. Difference of absorbancy at 1 min. and 6 min. is shown in Fig. 2.

10 50  $\mu$ M L-carnitine solution (50  $\mu$ l) was also treated in the same manner as described above. Then L-carnitine in the normal serum was calculated and was observed as 54.3  $\mu$ M.

Example 3

15 Reagent :

40 mM Glycine-NaOH buffer (pH 10.0)  
 5 mM Thio-NAD (Sankyo Co.)  
 0.5 mM NADH (Oriental Yeast Co.)  
 0.5 % Polyoxyethylene (20) sorbitan monooleate (Wako Pure Chem. Co.)  
 20 120 U/ml L-carnitine dehydrogenase (obtained from Ref. Example 2)  
 2 mM Oxamic acid

Operation :

The above reagent (1 ml) was put into cuvettes. Specimen (50  $\mu$ l), to which L-carnitine was added to normal serum in concentration of L-carnitine being 10, 20 and 50  $\mu$ M, was treated in the same manner as described  
 25 in Example 2. Result is shown in Table 1, where yield is observed as 97.0-102.0 %.

Table 1

Amount added ( $\mu$ M)	Observed ( $\mu$ M)	Difference ( $\mu$ M)	Yield (%)
0	54.3	—	—
10	64.5	10.2	102.0
20	74.2	19.9	99.5
50	103.3	48.5	97.0

Example 4

Reagent :

50 40 mM Glycine-NaOH buffer (pH 10.0)  
 5 mM Thio-NAD (Sankyo Co.)  
 0.2 mM NADH (Oriental Yeast Co.)  
 0.5 % Polyoxyethylene (20) sorbitan monooleate (Wako Pure Chem. Co.)  
 100 U/ml L-carnitine dehydrogenase (obtained from Ref. Example 2)  
 55 2 mM Oxamic acid

Operation :

2 mM Tris-HCl buffer (0.025 ml) and 1 N KOH (0.025 ml) were added to serum (0.05 ml). Acylcarnitine in serum was hydrolysed by incubating at 37°C for one hour. Thereafter the reaction mixture was neutralized by

adding 2.5 N HCl (0.05 ml) to prepare a specimen for total carnitine assay. A mixture of serum (0.05 ml) and physiological saline (0.1 ml) was prepared as a specimen for free carnitine assay.

The above each reagent (1 ml) was put into cuvettes, and a specimen hereinabove prepared (each 0.05 ml) was added to each cuvet respectively, then incubated at 37°C. After reaction was started, a difference at 1 min. and 6 min. In absorption at 400 nm was obtained.

L-carnitine with known concentration was treated in the same manner as above, and an amount of total carnitine and that of free carnitine were calculated from the observed value, then an amount of acylcarnitine was measured from a difference between amount of total and free carnitine.

In Table 2, result obtained from three different serums is shown.

Table 2

	Total carnitine ( $\mu$ M)	Free carnitine ( $\mu$ M)	Acylcarnitine ( $\mu$ M)
Serum 1	84.5	35.3	49.2
Serum 2	50.0	26.3	23.7
Serum 3	89.5	43.2	46.3

Although the present invention has been described in connection with various preferred embodiments thereof, it will be appreciated that these embodiments are provided solely for purposes of illustration, and should not be construed as limiting the scope of the invention. Other embodiments and applications of the invention will be readily apparent to those skilled in the art from reading the present specification and practicing the techniques described herein, without departing whatsoever from the scope and spirit of the appended claims.

#### Claims

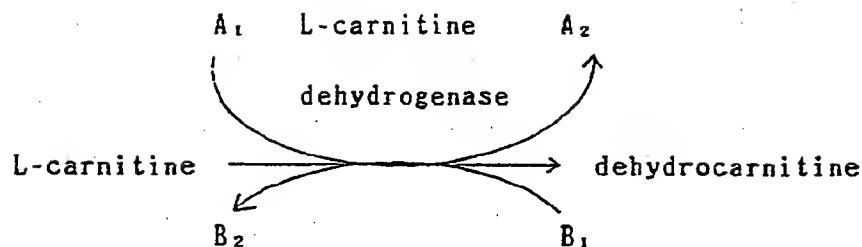
1. A method of assaying L-carnitine which comprises reacting a specimen comprising the L-carnitine to be assayed with reagents comprising :

① L-carnitine dehydrogenase which has coenzymes of the thionicotinamide adenine dinucleotide group (hereinafter designated as the thio-NAD-group) and nicotinamide adenine dinucleotide group (hereinafter designated as the NAD group) and which catalyses a reversible reaction forming essentially dehydrocarnitine from L-carnitine ;

② A<sub>1</sub> ; and

③ B<sub>1</sub> ;

to perform the cycling reaction :



wherein :

$A_1$  is the thio-NAD group or the NAD group ;

$A_2$  is a reduced form of  $A_1$  ;

when  $A_1$  is the thio-NAD group,  $B_1$  is a reduced NAD group and when  $A_1$  is the NAD group,  $B_1$  is a reduced thio-NAD group, and

$B_2$  is an oxidized form of  $B_1$  ;

and measuring the amount of  $A_2$  generated or  $B_1$  consumed.

2. A method according to claim 1 wherein the L-carnitine dehydrogenase has been obtained from the genus Alcaligenes.
3. A method according to claim 2 wherein the L-carnitine dehydrogenase has been obtained from Alcaligenes sp. No. 981 FERM BP-2570.
4. A method according to any one of the preceding claims wherein the NAD group is nicotinamide adenine dinucleotide (NAD), acetylpyridine adenine dinucleotide (acetyl NAD), acetylpyridine hypoxanthine dinucleotide or nicotinamide hypoxanthine dinucleotide (deamino-NAD).
5. A method according to any one of the preceding claims wherein the thio-NAD group is thionicotinamid adenine dinucleotide (thio-NAD) or thionicotinamide hypoxanthine dinucleotide.
6. A composition suitable for assaying L-carnitine which comprises components ① - ③ as defined in any one of the preceding claims.
7. L-carnitine dehydrogenase obtained from Alcaligenes sp. No. 981 FERM BP-2570.
8. Alcaligenes sp. No. 981 FERM BP-2570.

FIG. 1

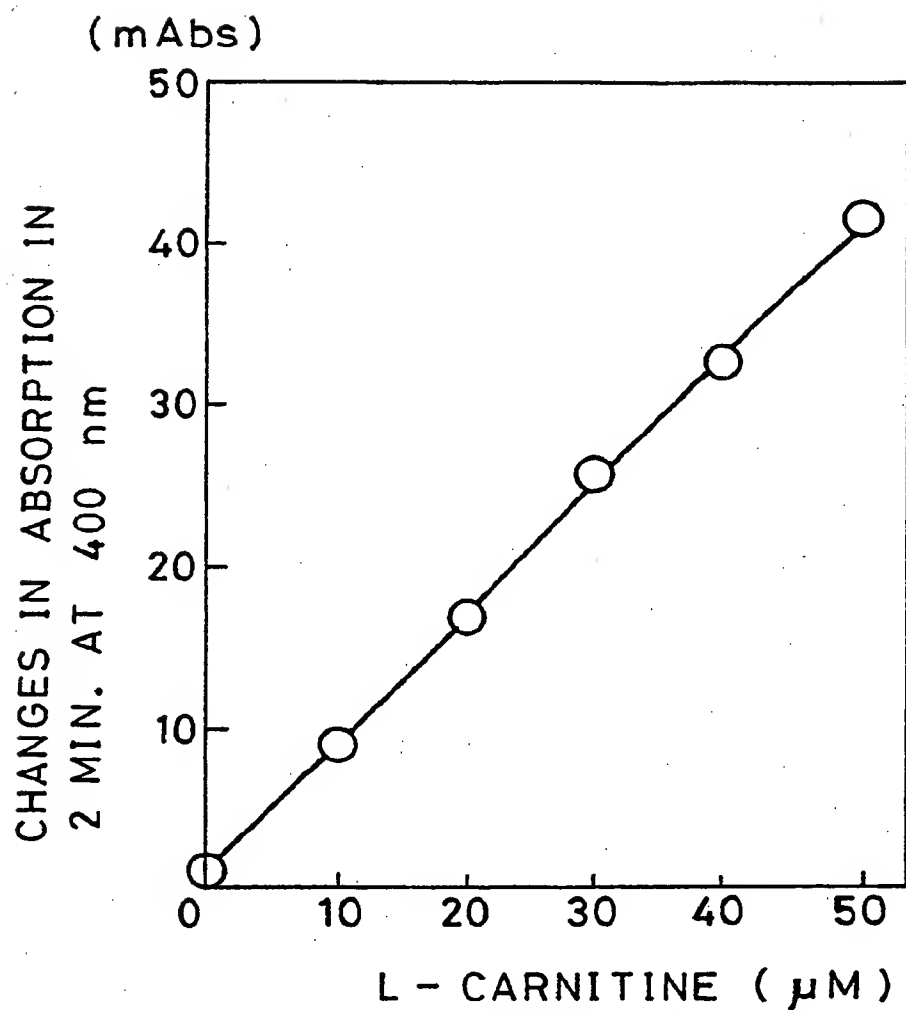
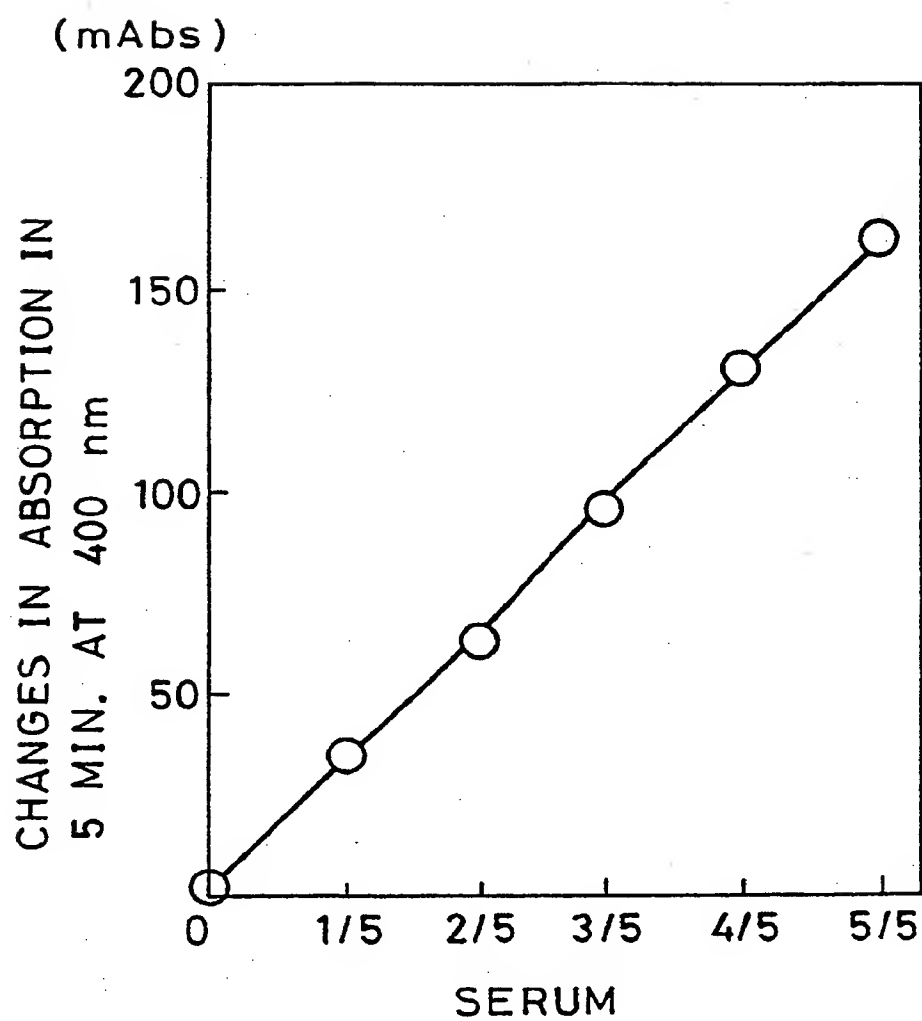


FIG. 2





European Patent  
Office

# EUROPEAN SEARCH REPORT

Application Number

EP 91 30 0201

DOCUMENTS CONSIDERED TO BE RELEVANT			
Category	Citation of document with indication, where appropriate, of relevant passages	Relevant to claim	CLASSIFICATION OF THE APPLICATION (Int. Cl.5)
Y	FR-A-2 398 046 (INSTITUT FRANCAIS DU PETROLE) * The entire document *	1,4-6	C 12 Q 1/26 C 12 Q 1/32 C 12 N 9/04 C 12 N 1/20 //
A	---	2,3,7,8	(C 12 N 9/04 C 12 R 1:05 )
Y	FR-A-2 596 865 (SOCIETE NATIONALE ELF AQUITAINE) * Page 2, line 32 - page 6, line 14; claims 1-5 *	1,4-6	
D, Y	--- FRESENIUS' ZEITSCHRIFT FÜR ANALYTISCHE CHEMIE, vol. 320, no. 3, March 1985, pages 285-289, Springer Verlag, Berlin, DE; W. SCHÖPP et al.: "Quantitative Bestimmung von L-Carnitin mit Hilfe von Carnitindehydrogenase aus Pseudomonas putida" * The entire document *	1,4-6	
			TECHNICAL FIELDS SEARCHED (Int. Cl.5)
			C 12 Q C 12 N
The present search report has been drawn up for all claims			
Place of search THE HAGUE		Date of completion of the search 13-03-1991	Examiner DOEPFER K-P.
<p><b>CATEGORY OF CITED DOCUMENTS</b></p> <p>X : particularly relevant if taken alone Y : particularly relevant if combined with another document of the same category A : technological background O : non-written disclosure P : intermediate document</p> <p>T : theory or principle underlying the invention E : earlier patent document, but published on, or after the filing date D : document cited in the application L : document cited for other reasons</p> <p>&amp; : member of the same patent family, corresponding document</p>			

EPO FORM 1501 (3.82) (P0401)

